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ATTORNEY'S DOCKET NUMBER
NU-467AX

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 36 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
09/869364

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/00470	7 January 2000	8 January 1999

TITLE OF INVENTION
ELECTRO-PNEUMATIC DISTRIBUTOR FOR MULTIPLEXED μ-TAS DEVICES

APPLICANT(S) FOR DO/EO/US
Barry L. Karger, Huanghui Liu, Frantisek Foret

Applicant herewith submits to the United states Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). UNSIGNED
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

Express Mail Number
EL751777570US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/869364	PCT/US00/00470	NU-467AX

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

		CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1,000.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$860.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 690.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	6 - 20 =	- 0 -	X **$18.00**	$ - 0 -	
Independent claims	3 - 3 =	- 0 -	X **$80.00**	$ - 0 -	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+**$270.00**	$ - 0 -	
TOTAL OF ABOVE CALCULATIONS =				$ 690.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$	
SUBTOTAL =				$ 345.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				**$ 345.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property				$	
TOTAL FEES ENCLOSED =				$ 345.00	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $ 345.00 to cover the above fees is enclosed. A check in the amount of $_____ is enclosed for the assignment recordation fee.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0804 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Customer Number 207

SEND ALL CORRESPONDENCE TO:

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Ten Post Office Square
Boston, Massachusetts 02109

Date: June 27, 2001

Holliday C. Heine
SIGNATURE

NAME Holliday C. Heine, Ph.D.

REGISTRATION NUMBER 34,346

9 pts

TITLE OF THE INVENTION

ELECTRO-PNEUMATIC DISTRIBUTOR FOR MULTIPLEXED μ-TAS DEVICES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the priority of U.S. Provisional Patent Application No. 60/115,167 filed, January 8, 1999 entitled ELECTRO-PNEUMATIC DISTRIBUTOR FOR MICROFABRICATED μ-TAS DEVICES, the whole of which is hereby incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

N/A

BACKGROUND OF THE INVENTION

Microfabricated systems, or microdevices, particularly multiplexed systems, with integrated channels for performing chemical analyses on a micro-scale level are an integral part of modern analytical methods. Such systems, frequently called Micro-Total-Analytical-Systems (μ-TAS), are expected to play a significant role in analytical and bioanalytical chemistry as well as in modern chemistry in general. Simultaneously, highly parallel structures are being developed for high throughput analyses. Although many structures can be completely integrated on the same microdevice, it is always necessary to use supporting

devices to communicate with the "macro-world." Additional supporting devices suitable for high throughput analyses would be highly desirable.

BRIEF SUMMARY OF THE INVENTION

The invention is directed to a universal electro-pneumatic distributor for supplying electric current and pressurized gas where needed, e.g., to microfabricated devices, and to methods for its use. The distributor of the invention is suitable for simultaneous, selective application of pressure and electric current, e.g., to individual channels of a microdevice, in a microfabricated μ-TAS system, so as to cause a fluid sample in an individual well in the surface of the device to flow in the associated individual channel and an electric current to flow across the channel. The function of the distributor of the invention is described here as a distributor assembly in conjunction with a microdevice for electrospray mass spectrometry, e.g., according to U.S. Patent No. 5,872,010, the whole of which is hereby incorporated by reference herein.

An electro-pneumatic distributor assembly for electrospray mass spectrometry can be attached to a linear computer controlled translation stage. When the system is in use, an individual channel exit port is aligned with the mass spectrometer sampling orifice, and gas pressure, e.g., is applied sequentially through a switching board coupled with the system. The switching board can also be used to connect the high voltage power supply to induce electrospray sample ionization. High throughput ESI/MS is achieved by application of both electrospray voltage and pressure sequentially to the

samples loaded in the individual sample wells in the microdevice. Sample throughput is maximized since a subsequent sample can be analyzed immediately after sufficient information is acquired from the previous one. There are barely any delays between the analysis of individual samples since no injection or washing steps are involved.

Alternatively, the system of the invention is for matrix assisted laser desorption ionization mass spectrometry. Such a system includes an interface having multiple deposition tips in conjunction with the electro-pneumatic distributor of the invention.

In another embodiment of the system of the invention, a liquid sample handling microdevice comprising an array of electrodes embedded in the device is associated with a pneumatic distributor that includes a microfabricated structure comprising an array of channels for gas transport. Preferably, the liquid sample handling microdevice is an electrospray interface having multiple electrospray tips, said electrospray interface further comprising an array of electrodes embedded in said interface, wherein individual electrodes in said array connect with individual said electrospray tips, and the microfabricated structure includes an array of channels for gas transport, said channels being oriented to permit application of pressure to selected individual electrospray tips of said interface.

The acceleration of drug discovery in recent years has presented significant analytical challenges. The number of compounds to be analyzed has increased dramatically since the introduction of combinatorial chemistry with automated parallel synthesis. High

throughput analytical techniques have become critical for determining the identity and purity of synthesized substances, as well as for clinical screening, pharmacokinetics and proteome related research.

Most of the current protocols for high throughput analysis are based on 96 (or larger) microtiter well plate technology where a large number of samples can be processed in parallel. The electro-pneumatic distributor assembly of the invention can be made compatible with the standard microtiter well plate technology format so that currently used sample processing procedures, such as solid phase extraction/desalting or enzyme digestion, can be combined on-line for complete, high throughput sample analysis.

BRIEF DESCRIPTION OF THE DRAWINGS

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

Fig. 1 is an exploded view of an electro-pneumatic distributor assembly of the invention;

Figs. 2A-2B show high throughput ESI-MS analysis using a plastic distributor system of the invention having 96 electrospray tips. (A) Cytochrome c and myoglobin solutions (5 μL) were alternately loaded into consecutive sample wells, and each well was analyzed every 5 seconds over a 40 sec time period. The concentrations for both proteins were 0.1 mg/mL. (B) Angiotensin II and angiotensin III solutions (5 μL) were

alternately loaded into the sample wells, and all 96 samples were analyzed as in (A). Concentrations of both peptides were 10 µg/mL;

Figs. 3A-3B show MS determination of HIV-1 protease inhibition using the system of the invention. (A) Relative signals of selected ion monitoring (SIM) spectra of the product tripeptide (Pro-Ile-Val; m/z =328 +/- 4) and the internal standard (Glu-Ile-Val; m/z =360 +/- 4) after incubation with increasing concentrations of pepstatin A (0-5µM). (B) Plot of data extracted from Fig. 3A; the IC_{50} was determined to be 0.75 µM with an RSD of 1.3%;

Figs. 4A-4B shows fabrication of a 96 ESI channel, 96 well microdevice for use in the system of Fig. 1, wherein Fig. 4A shows preparation of a silicone rubber negative imprint used for epoxy casting and Fig. 4B is a flow chart for device fabrication;

Fig. 5A is a micrograph of a microdevice for the system of the invention;

Fig. 5B is a detail of the microdevice of Fig. 5A showing sample wells connected to 300 µm wide semicircular distribution channels;

Fig. 5C is a detail of the microdevice of Fig. 5A showing an array of embedded electrodes for sequential connection of the electrospray high voltage; and

Fig. 6 is an exploded view of the system of the invention in position on a translation stage.

DESCRIPTION OF THE PREFERRED EMBODIMENT OF THE INVENTION

Mass spectrometry (MS) has become an indispensable tool for pharmaceutical research because of its

capability of sample identification, structure elucidation, quantitation and sensitivity. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most frequently used sample ionization techniques for automated high throughput MS analysis and are often coupled on-line with liquid chromatography (LC) or capillary electrophoresis (CE). Nevertheless, a significant portion of ESI-MS applications are also performed in the direct infusion mode. Typically, infusion ESI-MS is carried out with a flow injection (FIA) system equipped with an autosampler. Since every sample in such a system flows through the same conduit from the sampling probe through the injection valve to the ESI tip, the sampling probe must be carefully washed, and the flow conduit appropriately flushed to minimize sample cross contamination. Thus, useful mass spectrometric information can be observed only during a fraction of the total analysis time, leading to a low duty cycle. The electro-pneumatic distributor assembly of the invention is a qualitatively different approach to sample injection, permitting a significant improvement in performance with maximization of sample throughput.

Considering the wide acceptance of the microtiter well plate format in automated analysis and the potentially low cost of plastic devices, a disposable microdevice system equipped with an independent electrospray exit port for each sample well represents an attractive alternative to FIA. A microdevice with sample reservoirs positioned in the format of a standard

microtiter well plate can be used as the final receiving plate in a parallel sample processing scheme, such as selective enrichment, affinity capture, desalting, etc. The advantages of such a device compared to the standard FIA method include significantly simplified instrumentation, fast switching times for analysis of consecutive samples (high duty cycle) and elimination of sample cross contamination. The latter advantage, especially, leads to a significantly decreased number of runs required to validate that sample cross contamination did not occur.

Disclosed herein is a prototype plastic electro-pneumatic distributor, multisprayer device interfaced with a mass spectrometer for ESI-MS. Each of the sample wells was connected by an independent microchannel to a separate electrospray tip. All samples loaded onto the well plate could be analyzed in rapid sequence without the need for injection or washing. When coupled to a quadrupole ion trap mass spectrometer, all 96 sample wells could be scanned in 8 min, corresponding to a throughput as high as 720 samples/hr (5 sec per sample). Even shorter analysis times could, in principle, be obtained with a fast mass spectrometer, such as a time of flight instrument. It is important to note that, unlike in the case of flow injection, in the examples reported herein, a useful signal could be observed practically immediately and could be maintained as long as was needed (e.g., MS/MS) before advancing to the next sample.

The configuration of the electro-pneumatic distributor of the invention and its use in an electro-pneumatic distributor assembly for electrospray mass spectrometry will now be presented. Referring to Fig. 1,

an electro-pneumatic distributor assembly 5 includes an electro-pneumatic distributor 22, a gasket 18 and an electrospray microdevice 10. Electrospray microdevice 10 contains an array of individual sample wells 12 set in the device surface and an array 13 of electrospray tips 14 protruding from the side of the device. Each well 12 is connected through an independent channel 16 to an independent electrospray tip 14. A gasket 18, having an array of holes 20, is sandwiched between device 10 and electro-pneumatic distributor 22. Both the number of holes 20 in gasket 18 and the pattern of the holes are the same as those of wells 12 on microdevice 10. Gas flow channels 28, for supplying pressurized gas, and electrodes 24 are integrated within distributor 22. Electrodes 24, having opposite ends 25, 26, are arranged so that ends 25 of each electrode protrude from the undersurface of distributor 22 according to the format of the wells on microdevice 10. Electrode ends 25 are positioned so as to be in direct contact with the sample solutions in individual wells of device 10 when electro-pneumatic distributor assembly 5 is in use. Gas flow channels 28 have outlets 29 on the underside of distributor 22, which are also positioned according to the format of wells 12 on microdevice 10. The inlets 30 to gas flow channels 28, along with electrode contact ends 26, are positioned in separate linear arrays on the side of distributor 22, each array having the same spacing as that of electrospray tip array 13 on microdevice 10.

Electric current and pressurized gas are supplied to distributor 22 through electric conductor 32 and gas supply channel 34, respectively, situated in supply block

36, which is positioned against the side of electro-pneumatic distributor 22 and accessible to gas flow channel inlets 30 and electrode contact ends 26. Supply channel 34 is connected to a pressurized gas, e.g., nitrogen, and aligned with a gas flow channel inlet 30 on distributor 22. At the same time, electric conductor 32, to which high voltage is connected, is in communication with an electrode contact end 26 in distributor 22. Distributor 22 and microdevice 10 are brought together with gasket 18 sandwiched in between and then mounted on a translation stage (not shown).

The diameter of channels 16 connecting sample wells 12 with their respective electrospray tips is significantly larger (e.g., 300 μm) than the ESI tip inner diameter, e.g., at 26 μm. Therefore, the channel length, e.g., (1-8 cm) has an insignificant effect on the sample flow rate. Practically all flow resistance is due to the electrospray tip. After application of gas pressure and high voltage, the electrospray stabilizes in 1 sec, as can be observed by monitoring the total ion current. At the beginning of a run, the first of the 96 tips was aligned with the mass spectrometer sampling orifice, with the remaining tips being sequentially positioned at the orifice automatically by means of the fixed step movement of the stage controlled by the computer.

The system of the invention was first tested with an aqueous solution of 10 μg/mL angiotensin II at various pressures (3-40 psi) and voltages (2.5-7 kV), as well as at various distances between the ESI tip and the MS sampling orifice (1-8 mm). Based on the observed signal intensity and stability, settings of 5 psi, 4.5 kV and 3

mm were chosen for all further experiments. Under these conditions, the samples were electrosprayed at a flow rate of ~200 nL/min, i.e., within the optimum range for the capillary electrospray tip. With the motor and the motor driver used, the minimum time required to move from one channel to the next was 1 sec; however, much faster stages would be commercially available, if necessary.

The electro-pneumatic distributor system for ESI/MS analysis can be viewed as a logical extension of the microtiter well plate technology. All 96 (384, 1536 ....) samples deposited in a microtiter well plate can, in principle, be automatically processed (e.g., incubation, desalting, solid phase extraction, affinity capture, etc.) in parallel and finally deposited into the microfabricated device with electrospray tips, for rapid sequential MS analysis. Kinetics studies and multi-step analysis can be performed periodically for an individual sample in the well plate. During the interval of the analysis, the well plate can be taken away from the stage for further appropriate treatment of the samples. By combining parallel off-line SPE sample preparation with the multichannel device of the invention, sensitive and high throughput quantitation using ESI-MS can be realized (low ng/μL, sample/5 sec, RSD 13%).

The system of the invention is a disposable counterpart to standard microtiter well plate technology and should be useful in situations where throughput is a key factor, such as compound confirmation and purity estimation of combinatorial libraries, pharmacokinetics studies, substance aging testing, etc. Arranging the electrospray tips, electrodes or gas channels in 2-dimensional (or even 3-dimensional) arrays can further

increase density without increasing the size of the device.

Although the system of the invention can be made compatible with standard well plates, the dimension, density, geometry and pattern of the wells can be varied, as well as the orientation of channels connecting the wells to individual electrospray tips. Miniaturized, microfabricated devices may provide higher throughput for analysis, as appropriate. The number of wells in a microdevice is, in theory, unlimited. The volume of a well can range anywhere from, e.g., 0.1-2000 µl, and the channel diameter of an individual gas channel can be, e.g., 50-500 µm.

Using a computer controlled on the basis of the information from the mass spectrometer, an operator can continue mass spectrometer analysis for an individual channel as long as the sample in the well lasts. During this analysis period, the operation mode of the MS system can be varied (e.g., from full scan to single ion monitoring to MS/MS) to achieve the goal of the analysis. For example, if the sample is a synthetic library and the quality of the library is to be determined, the first determination would be MW. If there is no ambiguity, then another sample would be tested. If the structure in not clear from MW determination, a fragmentation would be carried out, with this decision being under computer control.

Thus, it can be seen that the system of the invention is suitable for any type of high throughput ESI-MS analysis. For example, after sample preparation or any other procedures are carried out on other systems, the samples can be transferred to the system of the

invention for ESI-MS analysis. As described in the Examples section, below, this system has been employed in HIV inhibitor studies using a synthesized peptide library. After reaction of a mixture of the peptides, substrate and the HIV protease, salts were removed through a solid phase extraction (SPE) process performed on a commercially available cartridge array in standard well plate format. Then, the sample was transferred to the system of the invention for high throughput analysis of the substrate and cleavage products.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE I

High throughput ESI/MS infusion analysis

In order to demonstrate the high throughput capability of the system, several sample solutions were alternately deposited in the wells and then analyzed sequentially and automatically. The spectra of cytochrome c and myoglobin from 8 consecutive channels are shown in Fig. 2A. Strong signals with well defined envelopes of the multiply charged protein ions were obtained every 5 seconds for each consecutive sample. Since fine electrospray capillary tips were used, the electrospray stabilized practically instantly, and no sample cross contamination was observed. If required, even smaller

diameter ESI tips (nanospray) could be used without modification of the basic device.

In a similar experiment shown in Fig. 2B, angiotensins II and III were electrosprayed in 8 minutes from all 96 wells, with singly charged ions of the two peptides being observed. The data demonstrate the validity of the approach to high throughput infusion analysis where all the samples loaded on the plate can be analyzed in a rapid sequence without risk of cross-contamination. Although several channels were blocked during the manual gluing of the device, it can be expected that this would be completely eliminated, if produced commercially. It is also worth noting that even higher throughput could be achieved with the use of a time of flight, instead of an ion trap mass spectrometer. Although, a detection level test was not included in this study, it is reasonable to expect the sensitivity to be equal to that achieved with single sprayer under the same conditions (tip dimension, sample flow rate, ESI voltage). Of course, the analysis may be programmed in such a way that the next sample is analyzed only after sufficient signal (information) is obtained. At a flow rate of 200 nL/min the sample consumption will be minimal even after extended data accumulation (minutes or more) and the unused samples may be used for additional studies, e.g. enzymatic digestion. Further improvements may also be expected by using a microfabricated array of electrospray tips instead of individual capillaries.

Besides higher throughput, the current device has additional advantages compared to ESI-MS analysis performed in the FIA mode. In the latter mode, the MS signal can be observed for only a limited time, as a

result of the fixed injected sample volume and flow rate. In the present system, the signal can be observed almost immediately and as long as desired, allowing a short time to acquire strong signals or a longer time to acquire weak signals of lower concentration samples. Switching to the next sample is not accompanied by any delays related to the system washing and sample injection. Furthermore, the sample amount consumed can be maintained as small as possible (e.g., ~15 nL or 150 fmol). Moreover, if necessary, practically all the sample deposited in the sample wells can reach the ESI tip and generate useful signal. This would be important with very low concentrated samples or when MS/MS analysis was necessary.

EXAMPLE II

HIV-1 Protease Inhibition Assay and IC50 Determination

The *in vitro* inhibition of HIV-1 protease was used as an illustration of the functionality of the high throughput system of the invention. The preparation of a series of samples with increasing concentration of the HIV-1 inhibitor (pepstatin A) is described in detail in Materials and Methods. Prior to ESI/MS analysis, 25 µL sample aliquots were desalted on a 96 well C_{18} solid phase extraction (SPE) plate. The substrate and standard, with no HIV-1 protease added, were also analyzed by direct infusion ESI-MS. No side product formation was observed, except Ser-Gln-Asn-Tyr(t-butyl)-Pro-Ile-Val (MW 875), which was expected from the substrate synthesis. This side product, however, had no influence in the present study since the m/z value was far removed from the internal standard (MW 359) and the enzymatically formed

tripeptide Pro-Ile-Val (MW 327). Fig. 3A presents selected ion monitoring (SIM) mass spectra with increasing amounts of inhibitor (pepstatin A), and the corresponding data are plotted in Fig. 3B. Inhibition by another peptidomimetic inhibitor N-Acetyl-Thr-Ile-Nle-ψ-[Ch2N]-Nle-Gln-Arg amine, MVT 101) and some other small organic molecules were also studied and the IC_{50} obtained are listed in Table 1. The experimental IC_{50} value of pepstatin A and the K_i value of MVT 101 were in agreement with those found in the literature within the experimental error, typical for this type of analysis (~ 20% or more).

Table 1. IC_{50} values of investigated HIV-1-protease inhibitors[a]

Inhibitor	Inhibitor Concentration Range (μM)	IC50 (this work) (μM)	IC50 (refs. ...) (μM)
Pepstatin A	0-5	0.75+/-0.1	0.55 μM
MVT 101	0-10	0.65 (K_i:~0.5μM)	K_i: 0.8 μM
Compound	0-12.5	9.5	-
Compound	0-40	6	-
Compound	0-30	24	-

[a] Assay conditions: 5 μL of 1 mg/mL HIV- 1 protease in a 100 μL total assay volume; incubation for 90 min at 37° C.

MATERIALS AMD METHODS

Fabrication of the Multi-Sprayer Microdevice

The 96 channel device was fabricated by casting from a solvent resistant polymer resin (EpoFix, EMS, Ft. Washington, PA), as shown in Figs. 4A-4B. The required patterns of channels and wells (master plates) were first created on rectangular plastic sheets (Lucite S-A-R,

Small Parts Inc., Miami Lakes, FL) using a digital milling machine. Second, the master plates were placed in a plastic box and silicone polymer (Silastic L-RTV silicone rubber kit, Dow Coming Corp., Midland, MI) was cast over the plates. Fig. 4A shows the fabrication of the silicone rubber negative with recessed channels of semicircular shape with diameter ~ 300 μm. Fig. 4B shows the complete flow diagram of the fabrication of the microdevice (only one of the 96 sample wells is depicted). The silicone negative imprints (c and d in Fig. 4B) of the Lucite master plates (a and b) were created, as described above. Master plate (a) contained 96 channels with starting points distributed in the standard 96 well plate pattern and ending in an array arrangement at the edge of the plate. The master plate (b) contained 96 wells with 5 mm diameter, 5 mm deep, connected to a 0.5 mm diameter 0.5 mm deep hole in the bottom. In the next step, both rubber imprints (c and d) were aligned to form a cavity, which was then filled with the liquid EpoFix resin. Two other polymeric resins were also tested: Acrylic-polyester based Casolite AP (AIN Plastics, Mt. Vernon, NY) and epoxy based Araldite (Fluka, Buchs, Switzerland); however, the EpoFix resin exhibited the best mechanical and chemical resistance properties. After hardening, the EpoFix part (e) was recovered and glued together with a bottom plate (f). The bottom plate, also prepared by casting, had 96 embedded electrodes (0.5 mm in diameter, 1.125 mm center to center distance). The electrodes were prepared from electrically conductive epoxy (Epo-Tek 415G, Epoxy Technology, Billerica, MA).

Finally, fused silica capillaries (2.5 cm in length, 26 μm i.d., 140 μm o.d.) were inserted into the exits of the channels to a depth of 1.5 cm and glued in place. About 1 mm of the polyimide coating at the capillary tips was removed by heat. This procedure produced a 96 well plate with closed channels and embedded electrodes connecting each well with a separate capillary electrospray tip, as can be seen in the micrograph of Fig. 5A.. The detail of Fig. 5B, at higher magnification, shows individual wells with their connected channels, and the detail of Fig. 5C shows an array of electrodes embedded into the channels just prior the attachment point of the electrospray tips.

An exploded view of the completed system in position on a translation stage is given in Fig. 6. The dimensions of the assembled electrospray were 16 cm x 10 cm x 0.9 cm.

Mass Spectrometry

An ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA), operated in the positive ion mode was used throughout this study. Since the sampling orifice of the instrument was located in a small hemispherical indentation, which cannot accommodate the size of the microdevice, an orifice extension was used to overcome the space restriction around the mass spectrometer inlet. The orifice extension was machined from an aluminum rod (2.5 cm long, 8 mm o.d.) with a 0.35 mm i.d. channel drilled axially. The extension was connected to the sampling orifice by a 2 cm long piece of silicone rubber tubing.

System Design and Operation

The exploded schematic diagram in Fig. 6 shows the total system design. During operation, the 96 well/96 ESI tips plate (sample plate) was positioned on a computer controlled translation stage so that the ESI tips were aligned with the MS sampling orifice extension. The sample plate was then closed by a pressure distribution plate. A thin sheet of silicone rubber with 96 properly positioned holes was placed between the two plates to seal the connection (not shown in Fig. 6).

Sequential sample flow through the ESI tips was initiated with the aid of a stationary gas pressure nozzle (200 µm i.d., 1 mm. o.d. Teflon tube) connected to a nitrogen tank. The nozzle contacted the surface of the pressure distribution cover plate so that channels were individually pressurized during the movement of the translation stage. The pressure distribution cover plate, with well and channel patterns as a mirror image of the sample well plate, was made by the same casting procedure as the sample plate. The stationary high voltage electrode (1 mm diameter stainless steel wire) was positioned so that the high voltage was connected only to the pressurized channel. The high voltage and nitrogen supply were applied during the course of analysis; as the translation stage moved the device to the next position, pressurized gas and high voltage were automatically connected to the respective sample well and channel. An aluminum plate was placed on top of the gas distributor to ensure gas tight sealing of all the wells. The linear translation stage (LS3-6-B 10, Del-Tron Precision, Inc., Bethel, CT) was driven by a NEMA 23 step motor controlled by a computer through a motor driver (6006-DB, American

Scientific Instrument Corp., Smithtown, NY). A simple computer routine (written in Basic) was used to control the translation stage.

Chemicals

Myoglobin, cytochrome c and angiotensins II, III, purchased from Sigma (St. Louis, MO), were each prepared at a concentration of 1 mg/mL and then diluted to the desired concentration with 0.2% (v/v) acetic acid in 50% (v/v) methanol. Fmoc-amino acids and H- val- 2-chlorotrityl resin were purchased from Anaspec (San Jose, CA). 1-hydroxybenzotriazol(HOBt), 2-(1H-benzotriazol-1,1,3,3 -tetramethyluronium) hexafluorophosphate (BBTU), diisopropylethylamine (DIEA), dimethylformamide (DMF), dichloromethane (DCM)], potassium cyanide, phenol, ninhydrin, pyridine and piperidine were obtained from Fluka (Ronkonkoma, NY). BPLC- grade acetonitrile (ACN) and methanol were also from Fluka. HIV- 1 protease was obtained from Pharmacia and Upjohn (Kalamazoo, MI) and pepstatin A and N-acetyl-Thr-Ile-Nle-ψ-[CH2N]-Nle-Gln-Arg amine (MVT 101) from Sigma. The organic compounds, 158393, 117027, 32180, were kindly donated by the Drug Synthesis & Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethseda, MD). Hack's balanced salt solution (HBSS) was obtained by Parker-Davis. Milli-Q water (Millipore, Medford, NL4,) was used throughout.

Sample Preparation for HIV-1 Protease Inhibition Assay

An 8-mer peptide substrate (Ser-Gln-Asn-Tyr-Pro-Ile -Val) and a 3-mer peptide internal standard (Glu-Ile-Val) were prepared, following procedure described in the Anaspec solid phase synthesis catalog (San Jose, CA). Peptide synthesis was begun from 0.5 mmol of

H-val-2-chlorotrityl resin, and coupling was performed by adding 1 mmol of FMOC amino acid in 1 mmol HBTU/HOBT, 2 mmol DIEA. The final peptide was then cleaved from the resin with a mixture of acetic acid/trifluoroacetic acid in dichloromethane and precipitated in ice cold ether. HIV-1 protease inhibition was measured by monitoring the concentration of the enzymatic degradation product - Pro-Ile-Val. The total assay volume was 100 µL, containing 50 µg/mL of HIV-1 protease, 1 mM substrate and a defined amount of inhibitor (pepstatin A or MVT 101) in a MES-buffer (100 mM MES, 300mM KCI, 5mM EDTA, 4.5% (v/v) DMSO, pH 5.5). The solution was incubated at 37° C for 90 min and then quenched by addition of 10 µL TFA. Finally, the solution was spiked with 600 µM of Glu-Val-Ile, the internal standard.

Aliquots of sample reaction products of 25-50 µL were taken and desalted on a 96 well C_{18} solid phase extraction (SPE) plate (Varian, Harbor City, CA). The plate was washed with 3x200 µL of methanol followed by 3x200 µL of water. The sample was introduced on the resin and washed extensively (4x 300 µL acidified water (10% (v/v) formic acid)). The sample was then eluted from the SPE resin with 3x 20 µL 1% (v/v) formic acid in 50% (v/v) ACN/$H_2$0. The eluate solutions were used for direct infusion or were stored in Eppendorf vials at -15° C for future analysis.

OTHER EMBODIMENTS

As described herein, the multiplexed µ-TAS system of the invention is particularly useful for electrospray-mass spectrometry analysis (ESI/MS). The system of the invention may also be used for atmospheric pressure-

chemical ionization mass spectrometry (APCI/MS), for matrix assisted laser desorption ionization mass spectrometry (particularly in a Time-Of-Flight instrument), for nuclear magnetic resonance analysis (NMR), for pneumatically or ultrasonically assisted spray sample handling, for transfer to an off-chip detection system, such as electrochemistry, conductivity or laser induced fluorescence, or for collection of specific fractions, e.g., in collection capillaries or on collection membranes. Sample transfer may be by droplet, spray or stream, as desired, or as suitable for the instrument or device receiving the transferred sample. The transferred fluid may be in the form of a liquid or a gas.

While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definitions contained in the appended claims and equivalents thereof.

CLAIMS

What is claimed is:

1. An electro-pneumatic distributor comprising

a microfabricated structure having an integrated array of channels for gas transport and electrodes, said channels and electrodes being oriented to permit simultaneous or sequential application of pressure and electric current to selected entrance ports of a device external to said structure.

2. A microfabricated μ-TAS system comprising

a fluid sample handling microdevice having multiple channels; and

the electro-pneumatic distributor of claim 1, for the simultaneous or sequential application of electric current and pressure to individual said channels of said sample handling microdevice.

3. An electrospray system for a mass spectrometer, said system comprising

an electrospray interface having multiple electrospray tips;

the electro-pneumatic distributor of claim 1, for supplying pressure and electric current simultaneously to individual electrospray tips of said interface; and

a gasket in between said interface and said distributor.

4. A matrix assisted laser desorption interface system for a mass spectrometer, said system comprising

a deposition interface having multiple deposition tips;

the electro-pneumatic distributor of claim 1, for supplying pressure and electric current simultaneously to individual deposition tips of said interface; and

a gasket in between said interface and said distributor.

5. An electrospray system for a mass spectrometer, said system comprising

an electrospray interface having multiple electrospray tips, said electrospray interface further comprising an array of electrodes embedded in said interface, wherein individual electrodes in said array connect with individual said electrospray tips;

a pneumatic distributor comprising

a microfabricated structure comprising an array of channels for gas transport, said channels being oriented to permit application of pressure to selected individual electrospray tips of said interface; and

a gasket in between said interface and said distributor.

6. An matrix assisted laser desorption interface system for a mass spectrometer, said system comprising

a deposition interface having multiple deposition tips, said deposition interface further comprising an array of electrodes embedded in said interface, wherein individual electrodes in said array connect with individual said deposition tips;

a pneumatic distributor comprising

a microfabricated structure comprising an array of channels for gas transport, said channels being oriented to permit application of pressure to selected individual deposition tips of said interface; and

a gasket in between said interface and said distributor.

SUBSTITUTE SHEET (RULE 26)

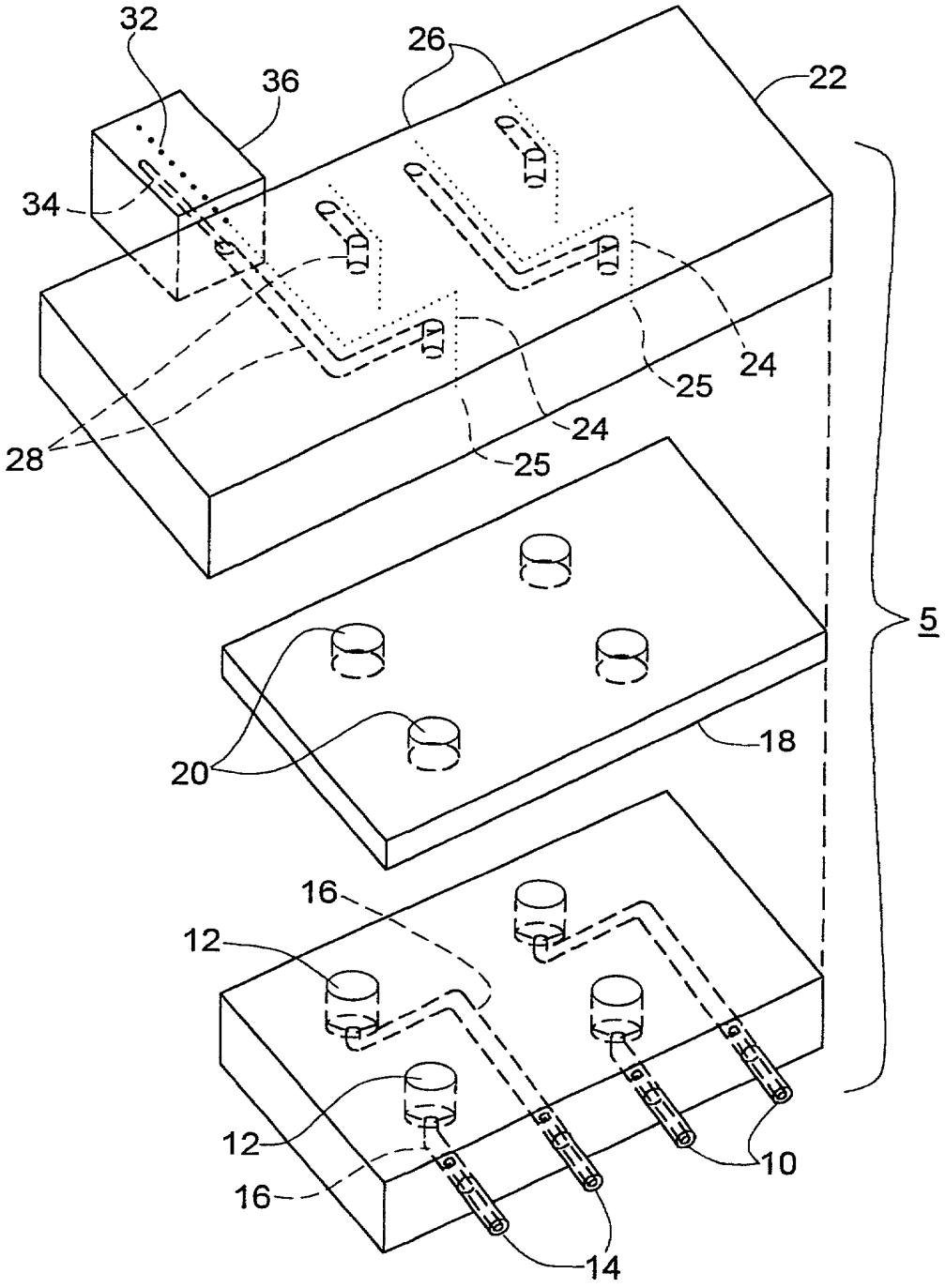

FIG. 1

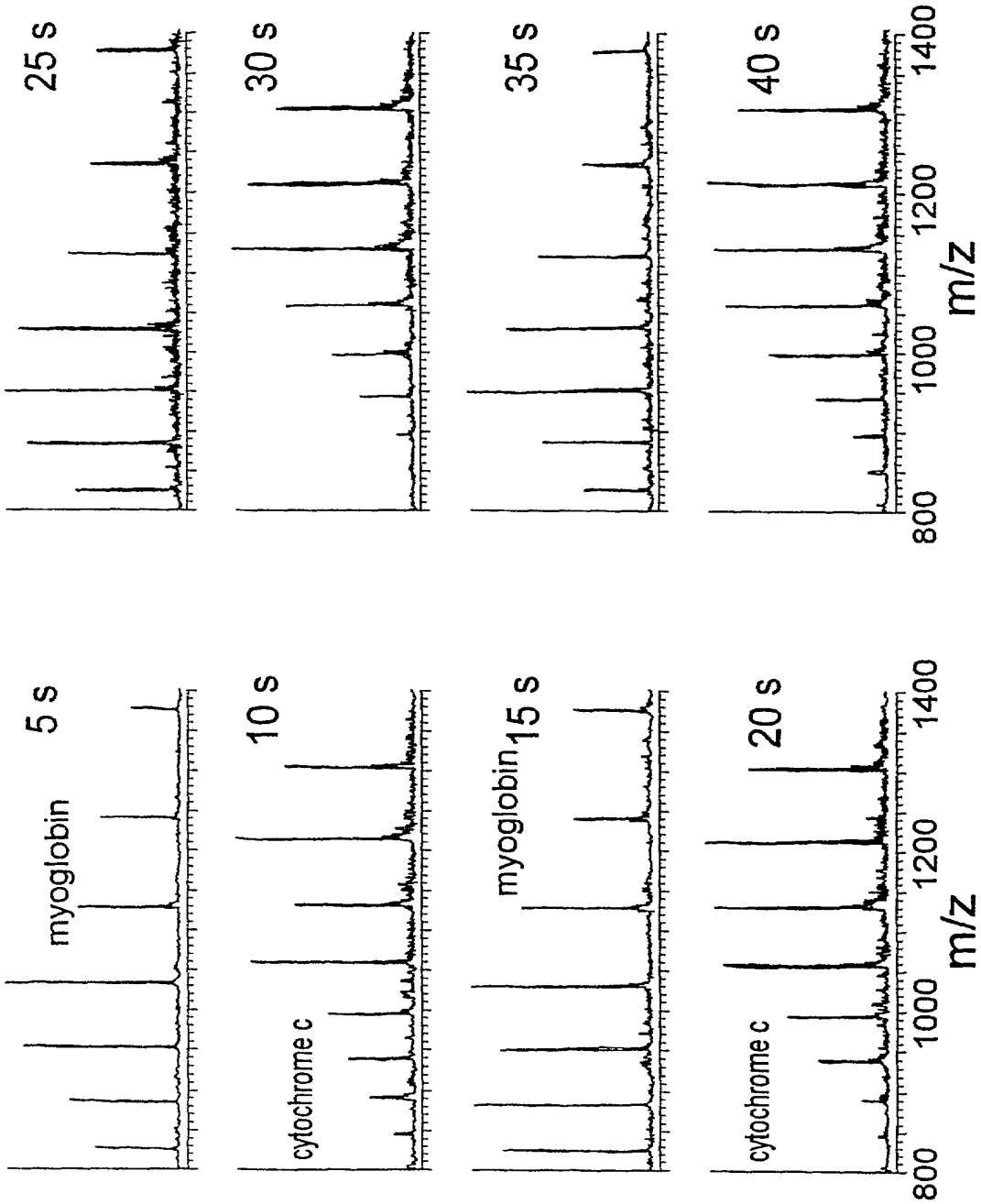
5 s
myoglobin
10 s
cytochrome c
15 s
myoglobin
20 s
cytochrome c
25 s
30 s
35 s
40 s
800
1000
1200
1400
m/z
RELATIVE SIGNAL INTENSITY

FIG. 2A

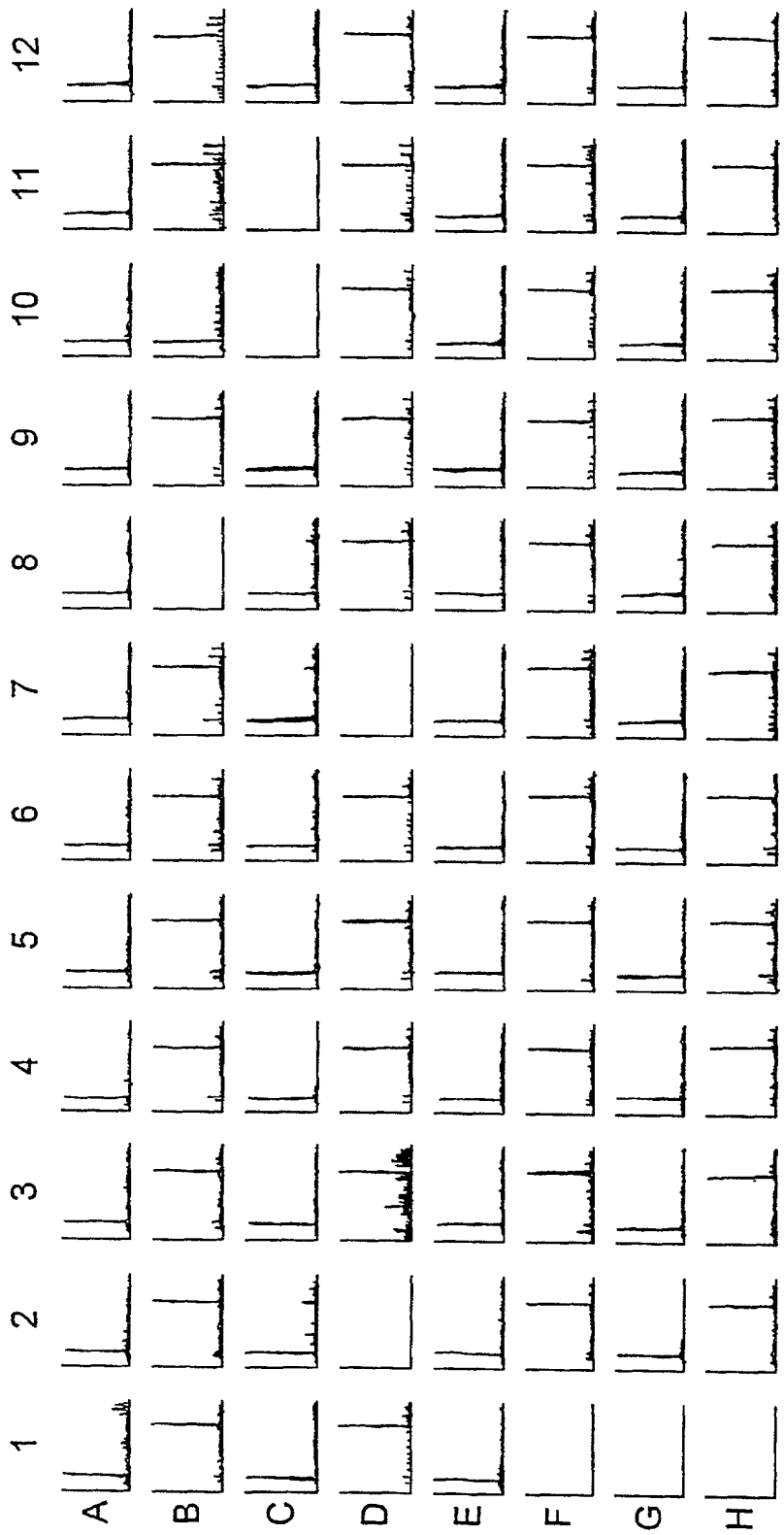

FIG. 2B

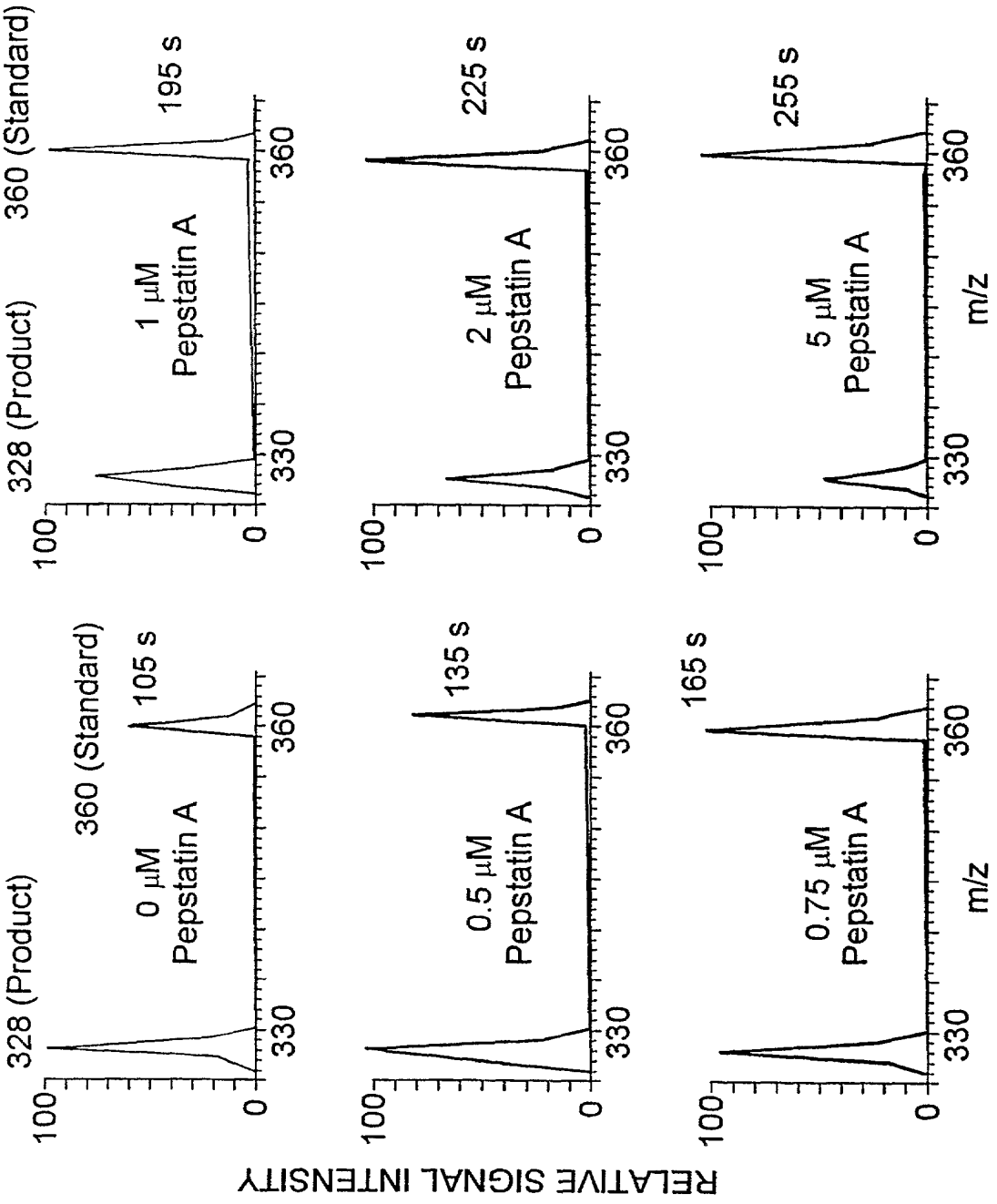
RELATIVE SIGNAL INTENSITY
328 (Product)
360 (Standard)
0 μM Pepstatin A
105 s
0.5 μM Pepstatin A
135 s
0.75 μM Pepstatin A
165 s
1 μM Pepstatin A
195 s
2 μM Pepstatin A
225 s
5 μM Pepstatin A
255 s
100
0
330
360
m/z

FIG. 3A

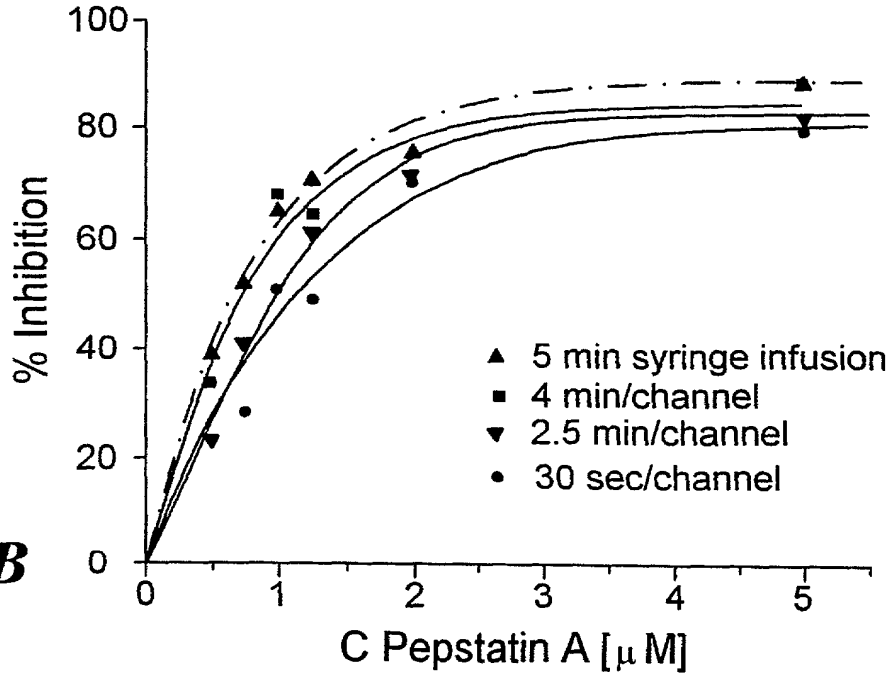
100
80
60
40
20
0
% Inhibition
0
1
2
3
4
5
C Pepstatin A [μM]
5 min syringe infusion
4 min/channel
2.5 min/channel
30 sec/channel

FIG. 3B

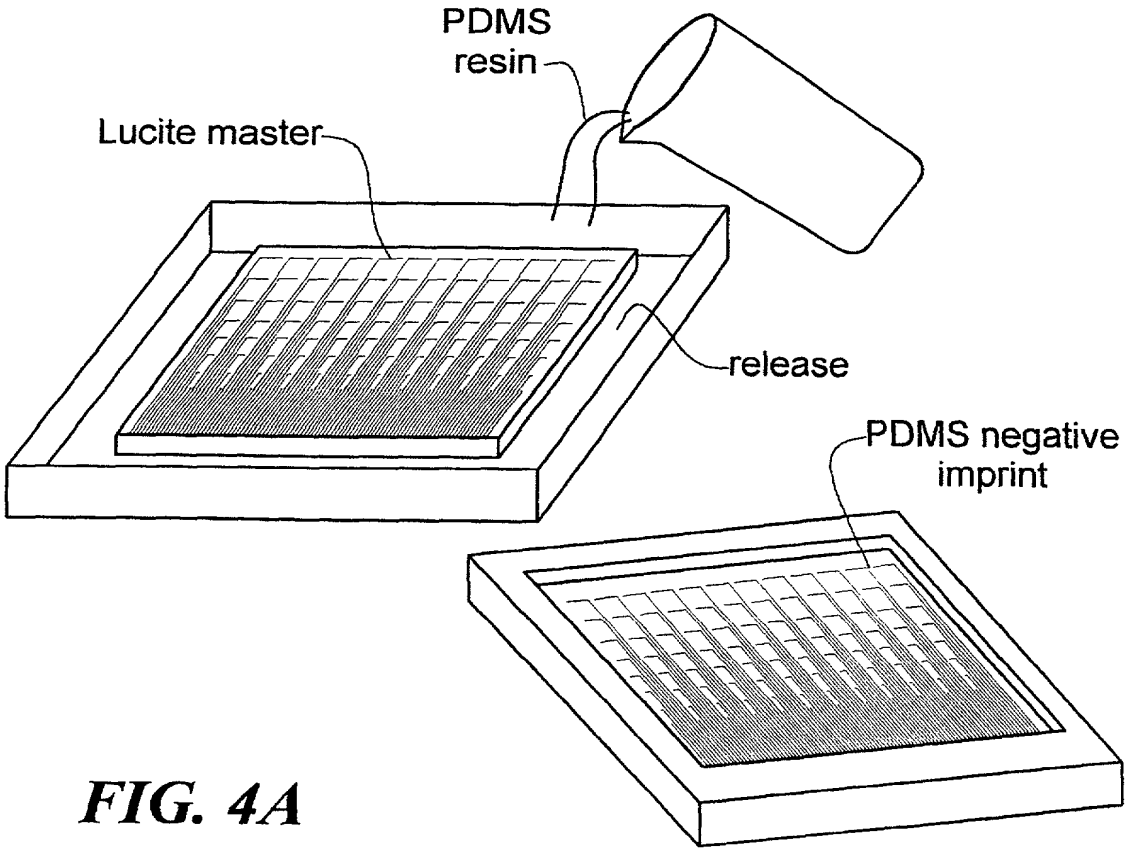
PDMS
resin
Lucite master
release
PDMS negative
imprint

FIG. 4A

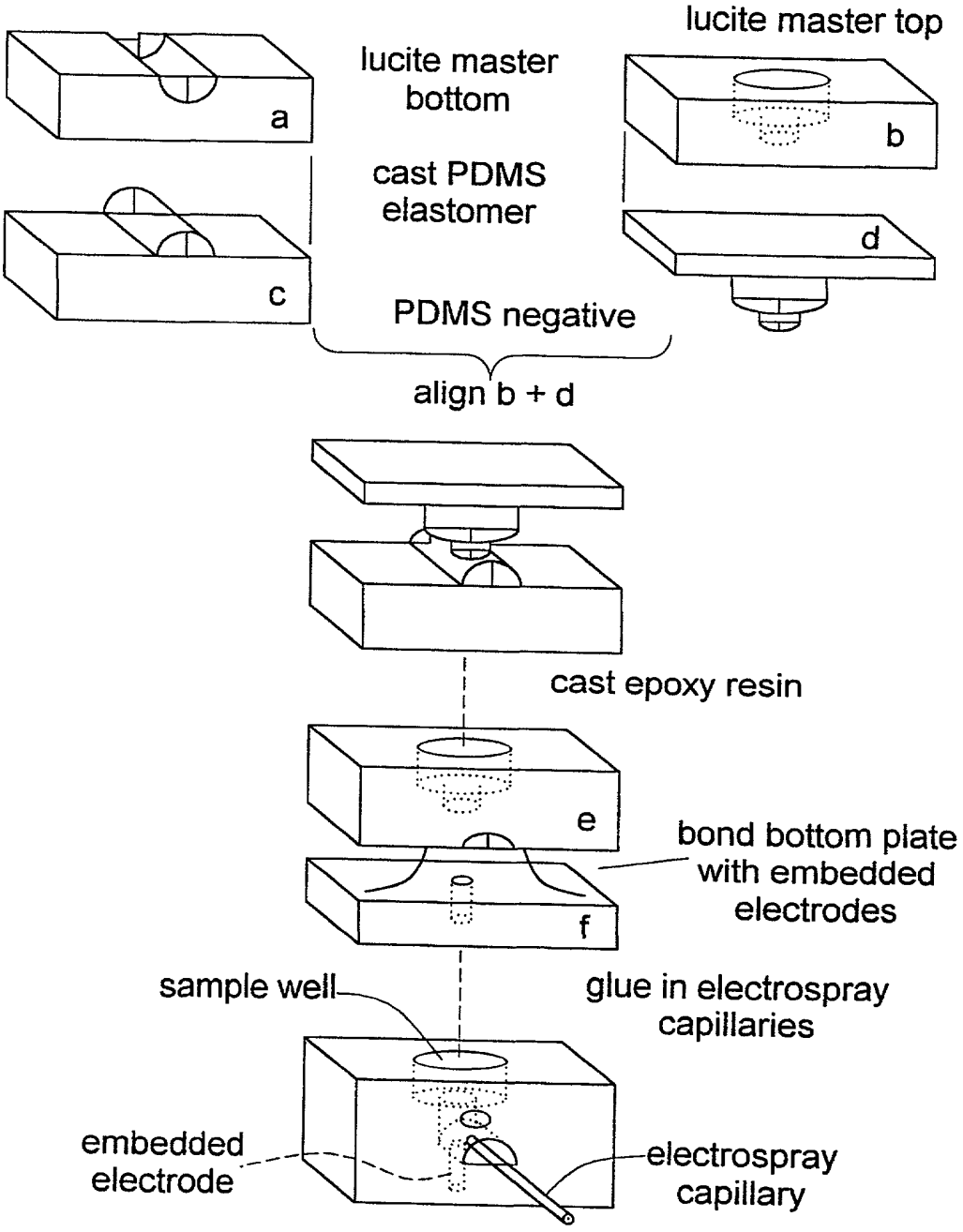
lucite master top
lucite master bottom
a
b
cast PDMS elastomer
c
d
PDMS negative
align b + d
cast epoxy resin
e
bond bottom plate with embedded electrodes
f
sample well
glue in electrospray capillaries
embedded electrode
electrospray capillary

FIG. 4B

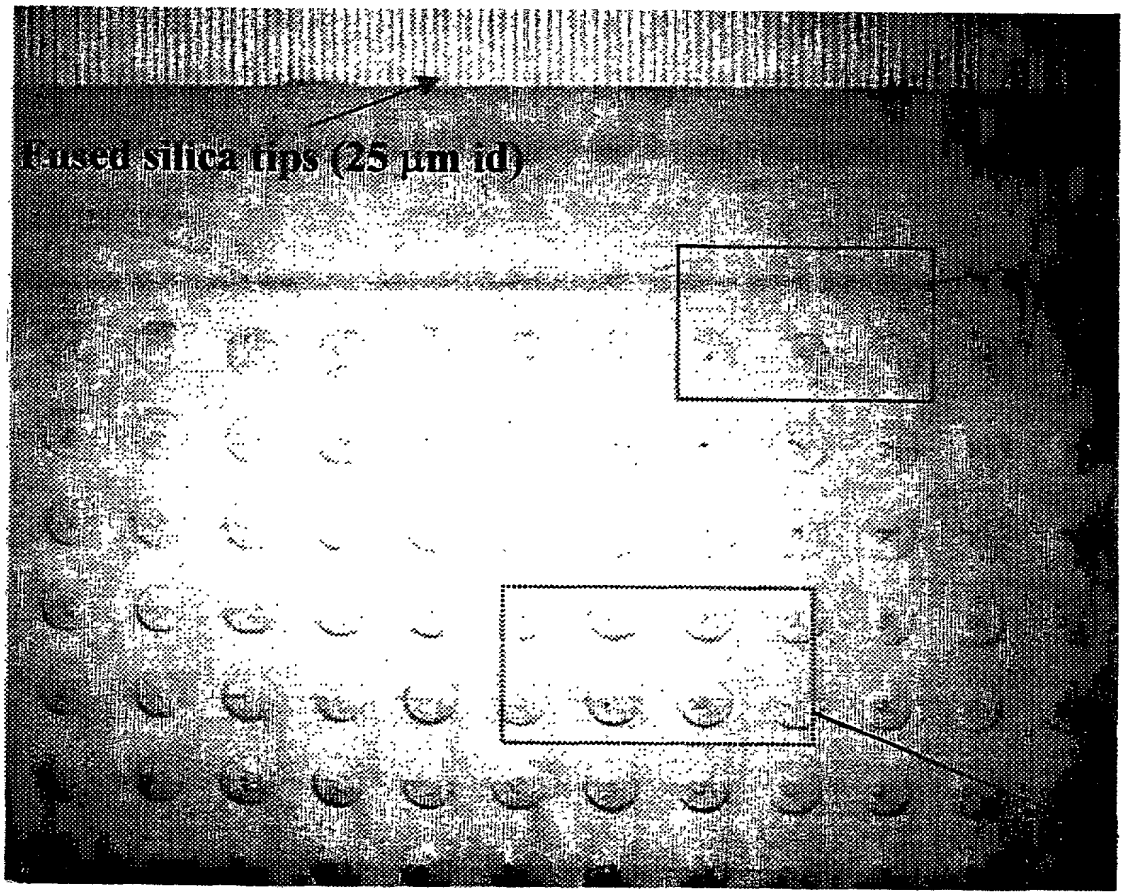

FIG. 5A

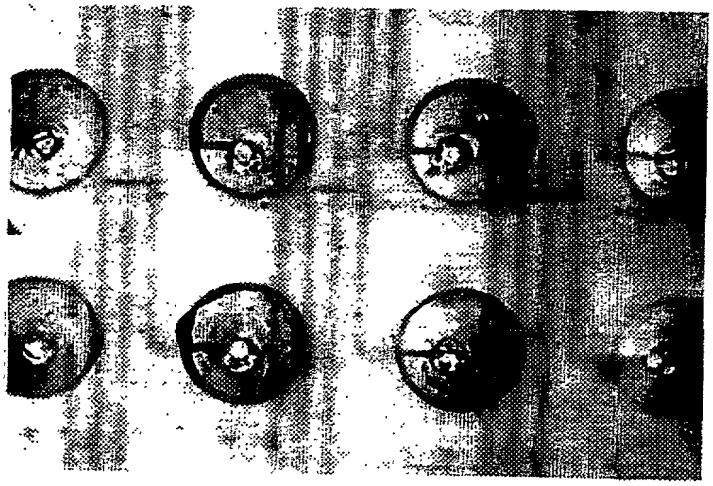

FIG. 5B

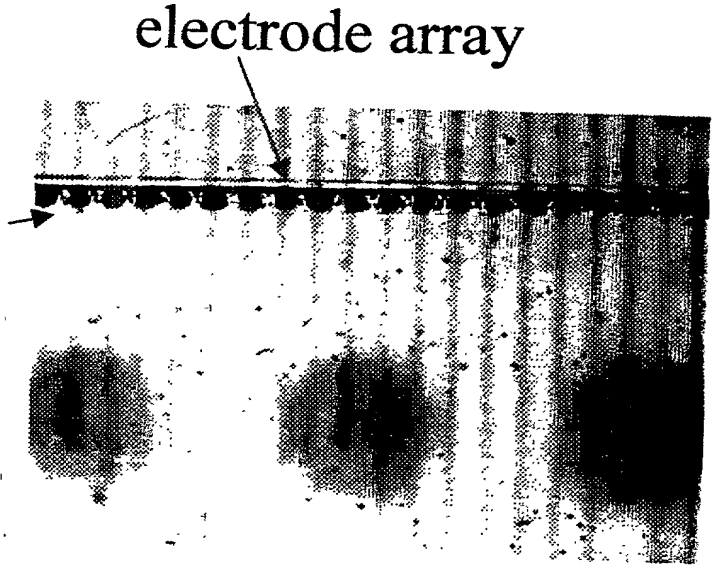

FIG. 5C

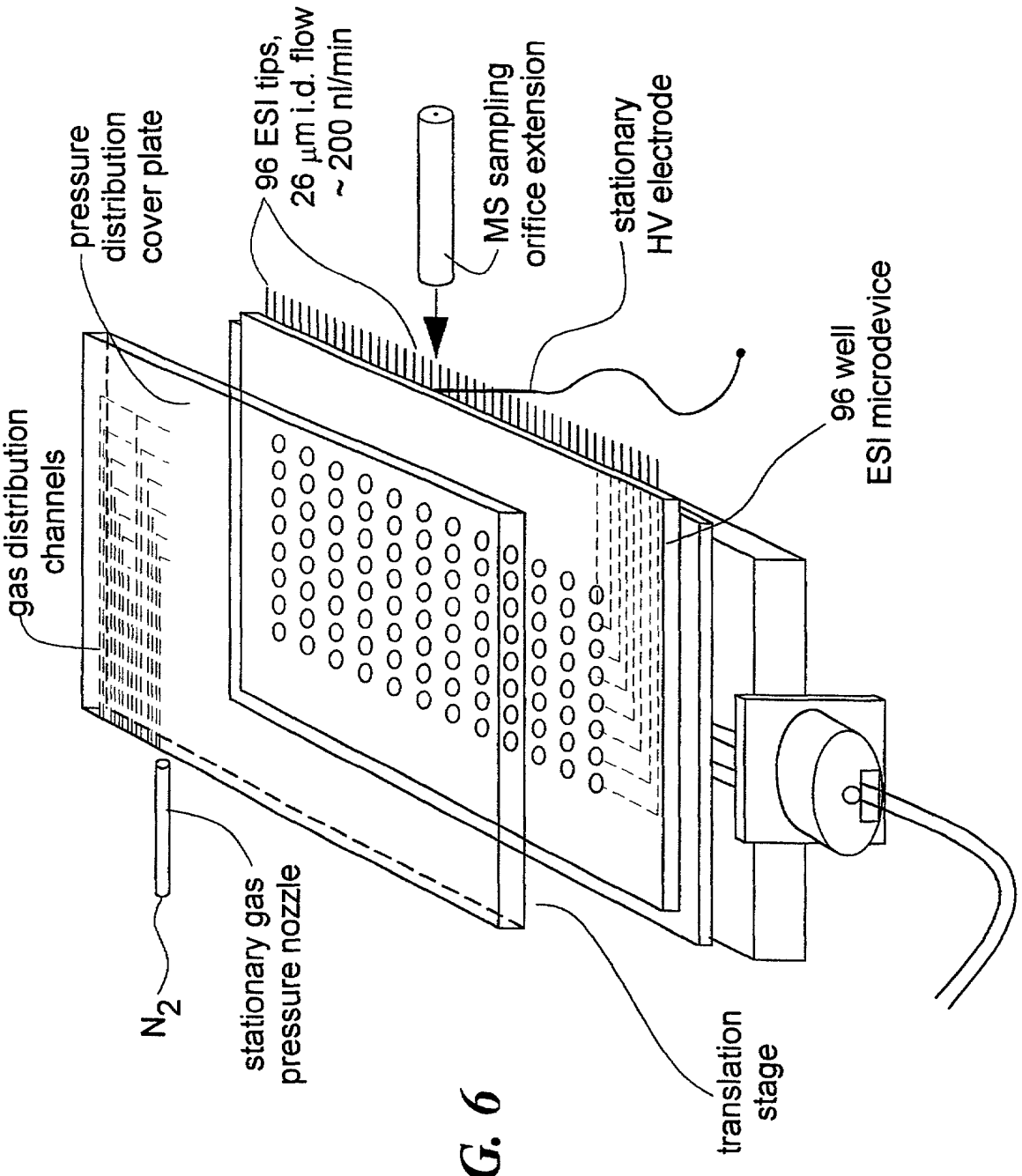
pressure distribution cover plate
96 ESI tips, 26 μm i.d. flow ~ 200 nl/min
MS sampling orifice extension
stationary HV electrode
96 well ESI microdevice
gas distribution channels
N_2
stationary gas pressure nozzle
translation stage

FIG. 6

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Attorney
Docket No.: NU-467AX

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ELECTRO-PNEUMATIC DISTRIBUTOR FOR MULTIPLEXED μ-TAS DEVICES

The specification of which (check one):

[•] **is attached hereto.** [ ] **was filed on** _____ **as Application No.** 09/869,364
amended on _____ **(if applicable).**

[X] was filed as PCT International Application No. PCT/US00/00470 on 7 January, 2000, and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, USC §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)		Date Filed	Priority Claimed	
_____ (Number)	_____ (Country)	_____ (Day/Month/Year)	[ ] Yes	[ ] No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year)	[ ] Yes	[ ] No

I hereby claim the benefit under Title 35, USC §119(e) of any United States provisional application(s) listed below:

60/115,167 (Application Number)	January 8, 1999 (Filing Date)
_____ (Application Number)	_____ (Filing Date)
_____ (Application Number)	_____ (Filing Date)

Express Mail Number

EL751777761US

Attorney Docket No.: NU-467AX

I hereby claim the benefit under Title 35 USC §120 of any United States application(s) listed below and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35 USC §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application No.)	(Filing Date)	(Patented/pending/abandoned)
(Application No.)	(Filing Date)	(Patented/pending/abandoned)
(Application No.)	(Filing Date)	(Patented/pending/abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business connected therewith in the Patent and Trademark Office, and to file with the USRO any International Application based thereon.

Stanley M. Schurgin, Reg. No. 20,979
Charles L. Gagnebin III, Reg. No. 25,467
Paul J. Hayes, Reg. No. 28,307
Victor B. Lebovici, Reg. No. 30,864
Eugene A. Feher, Reg. No. 33,171
Beverly E. Hjorth, Reg. No. 32,033
Holliday C. Heine, Reg. No. 34,346
Gordon R. Moriarty, Reg. No. 38,973
James F. Thompson, Reg. No. 36,699

Address all correspondence to:

WEINGARTEN, SCHURGIN, GAGNEBIN & HAYES LLP
Ten Post Office Square
Boston, Massachusetts 02109
Telephone: (617) 542-2290
Telecopier: (617) 451-0313

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First Inventor: Barry L. Karger		
City of Residence: Newton	State or Country: Massachusetts	Country of Citizenship: United States
Post Office Address: 62 Deborah Road	City: Newton	State or Country: Massachusetts Zip Code: 02459
Signature: (Please sign and date in permanent ink.) X Barry L. Karger		**Date signed:** X 7/9/01

Full Name of Second Inventor: HCH 8/27/01 2-00 Huanghui Liu		
City of Residence Somerville NA	State **or** Country Massachusetts	Country of Citizenship United States
Post Office Address 26 Pearl Street, Apt. 3	City Somerville	State **or** Country Zip Code Massachusetts 02145
Signature: (Please sign and date in permanent ink.) X Huanghui Liu		**Date signed:** X 07-18-01

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Attorney
Docket No.: NU-467AX

Full Name of Third Inventor:			
Frantisek Foret			
City of Residence Malden MA	State **or** Country Massachusetts	Country of Citizenship Czech Republic	
Post Office Address 525 Highland Avenue, Apt. # 40	City Malden	State **or** Country Massachusetts	Zip Code 02148
Signature: (Please sign and date in permanent ink.) **X** Frantisek Foret		**Date signed:** **X** 7/9/01	

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